Studies on the Extraction and Composition of Rice Endosperm Glutelin and Prolamin[1]

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ABSTRACT

Various extraction procedures were compared for the preparation of glutelin from milled rice (variety IR8). Extraction of protein with 0.015N sodium hydroxide before fractionation resulted in glutelin with 6% lysine. Glutelin preparations extracted after removal of albumin-globulin and prolamin had aminograms similar to those of whole milled-rice protein. Subfractionation of rice glutelin in the presence of beta-mercaptoethanol or detergent gave products differing widely in amino acid composition and electrophoretic mobility. Although glutelin had a molecular weight of 6×10^5, as measured by gel filtration, reduced glutelin showed a second fraction with a molecular weight of 6×10^4. Prolamin prepared by ethanol extraction had a low protein content and a high UV absorption which are indicative of polyphenols. The UV-absorbing contaminant dissociates from the prolamin in an alkaline solution and is more soluble in ethanol than prolamin. Acetone precipitation from the ethanol extract resulted in a pure prolamin preparation.

The proteins of cereals that are insoluble in water and in neutral salt solutions are glutelin and prolamin. Approximately 80% of the protein of the rice endosperm belongs to the type soluble only in dilute acid or alkali and is termed glutelin (1). Glutelin of rice is protein of high molecular weight (MW) composed of subunits bound by disulfide linkages (2). Rice protein exists in the endosperm in bodies 1 to 3 μ in size (3). Presumably, the ratio of protein fractions in the protein bodies of the endosperm is similar to that of milled-rice protein, since Mitsuda et al. (3) found that the glutelin content of a crude preparation was 83% of total protein. Various extractants were surveyed in our study to find solvents with pH values below 10 that can be used to obtain undegraded glutelin. Degradation of the cystine residues of corn glutelin has been observed above pH 10 (5).

Attempts have been made to relate the properties of glutelin proteins of other cereals to those of the more soluble forms—albumin, globulin, or prolamin (6,7,8). Since several methods for preparing rice glutelin exist, the properties of the glutelins were compared in the variety IR8. Subfractionation and reductive alkylation experiments were also undertaken to obtain information on the subunit proteins of rice glutelin.

Rice protein has one of the lowest (5%) contents of the alcohol-soluble or prolamin fraction among cereal proteins (1). Rice prolamin is characterized by a low protein purity and by carbohydrate contamination (glucan) (9). It is unusually low in proline, but is similar to prolamins of other cereals in being low in lysine and tryptophan. An attempt was also made to compare various methods of prolamin preparation and purification.

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MATERIALS AND METHODS

Samples of IR8 and BPI-76 rough rice were obtained from the Institute experimental farm, dehulled with a McGill sheller, and milled with a McGill miller No. 3. The resulting milled rice was ground into a 40- or 100-mesh powder with a Wiley mill. The flours were defatted twice with petroleum ether and air-dried before extraction.

Single-Extraction Experiments

The procedure of Cagampang et al. (1) for single extractions was followed. Milled BPI-76 rice flour (100-mesh, 100 mg.) was shaken mechanically with 2 ml. of solvent for 6 hr. at 25°C. and centrifuged at 500 × g. The residue was washed three times with distilled water, and micro-Kjeldahl nitrogen (10) was determined from a portion of the combined extract and washings.

Glutelin Preparations

Maes Percolation. The procedure of Maes (11) as employed by Cagampang et al. (1) was followed. IR8 flour (40-mesh, 400 g.) was mixed with 200 g. Celite filter-aid and packed into a 30 × 5-cm. column. It was leached with 0.015N and then 0.1N NaOH. The extracts were acidified and the precipitates washed with 60% ethanol.

Osborne Procedure. The procedure of Cagampang et al. (1) was followed. IR8 rice powder was extracted with a solution of 5% NaCl to remove albumin-globulin and a solution of 60% ethanol to remove prolamin. The residual rice was extracted with 0.05N NaOH and the glutelin was precipitated by acidifying with hydrochloric acid to pH 5.

Alkali Extraction. The method of Lózsa and Koller (12) was followed. Milled IR8 rice flour (170 g.) was soaked in 170 ml. water for 1 hr., then blended at medium speed in a Waring Blendor for 15 min., after which 170 ml. of 0.03N NaOH was added. The suspension was further stirred for 18 min. at low speed and centrifuged at 37,000 × g for 10 min. The supernatant fluid was neutralized to pH 5 with 6N hydrochloric acid and the protein precipitate was washed with 5% NaCl solution until the washing was negative to the biuret test for proteins. The prolamin was extracted from the washed residue with 60% ethanol. The glutelin residue was redissolved in 0.015N NaOH, reprecipitated by acidification, and washed with ethanol.

Reduction of Glutelin. Glutelin prepared by the Osborne method was further purified by the method of Sawai and Morita (13) and reduced and alkylated according to Beckwith et al. (14). The glutelin was dissolved in 0.05N NaOH, precipitated by acidifying with 6N hydrochloric acid, and centrifuged at 37,000 × g for 5 min. The supernatant fluid was carefully removed with a pipet. The activated glutelin was dispersed (without exposure to air) in a solution of 0.1M tris-maleate buffer (pH 8) containing 8M urea and then reduced under nitrogen gas with a 500-fold excess of beta-mercaptoethanol for 24 hr. with stirring. The glutelin dissolved completely in the reaction mixture. Acrylonitrile (2 moles per mole of mercaptoethanol) was added and after 45 min., the reaction mixture was adjusted to pH 3.5 with glacial acetic acid, dialyzed against distilled water, and lyophilized.

Subfractionation of Glutelin

IR8 milled rice (100-mesh flour, 8.33% glutelin), previously extracted with 5% NaCl and 70% ethanol solutions by the Osborne method, was used in the single-extraction experiments as described above for milled rice by the procedure of Cagampang et al. (1).

For the subfractionation study, the milled-rice glutelin was extracted in series with three solvents: three times with 70% ethanol containing 0.6% beta-mercaptoethanol three times with 0.5M NaCl in 0.1M sodium borate buffer (pH 10) with 0.6% beta-mercaptoethanol, and three times with 0.5M NaCl in 0.1M sodium borate buffer (pH 10) with 0.5% sodium dodecyl sulfate (6). The combined extracts for each solvent were dialyzed and lyophilized.

In another experiment, the milled-rice glutelin was extracted for 24 hr. under nitrogen gas with a 150-fold excess beta-mercaptoethanol in 0.1M tris-maleate buffer (pH 8) containing 3M urea. The reaction was stopped with acrylonitrile (twice the molar concentration of beta-mercaptoethanol) and the extract was separated by centrifugation, dialyzed, and lyophilized.

Prolamin Preparations

Prolamin was estracted directly from IR8 and BPI-76 milled rice with 70% ethanol. Ethanol was removed under reduced pressure in a rotary evaporator. The aqueous suspension was made 0.01N in NaOH and concentrated by ultrafiltration in a Diaflo model 401 cell with a PM-10 membrane. The filtrate was saved. In another experiment, prolamin was precipitated from the 70%-ethanol extract by the addition of acetone.

Prolamin was extracted with 60 to 70% ethanol from the IR8 residue of the albumin-globulin extraction in the Osborne method and the Maes percolation method. Further purification of prolamin was attempted by ultrafiltration.

Gel Filtration

Glutelin was chromatographed on columns (35 X 2.5 cm.) of Sephadex G-100 and G-200 and Sepharose 4B using as eluant 0.01M phosphate buffer (pH 7.0) containing 0.5M NaCl and 3M urea. Three-milliliter fractions were collected and their absorbance at 280 nm., protein content by the method of Lowry et al. (15), and polysaccharide content by the phenol-sulfuric acid method (16) were determined. Prolamin was dissolved in 0.01N NaOH and subjected to gel filtration on columns of Sephadex G-200 and eluted with 0.01N NaOH, or on columns of Sephadex LH-20 and eluted with 70% ethanol. Prolamin was also subjected to gel filtration on Bio-Gel P-300 with 0.01M phosphate buffer (pH 7.0) containing 3M urea as eluant. The columns were calibrated with crystalline proteins of known MW (9).

Electrophoresis

Starch-gel electrophoresis in the presence of urea was performed according to a modification of the procedure of Beckwith et al. (14). The amount of Connaught hydrolyzed starch recommended by the manufacturer for each batch was increased by 18 mg. per ml. of buffer used. Urea (3M) was added to the aluminum lactate buffer (0.008M aluminum lactate and 0.02M lactic acid, pH 3.1). Electrophoresis was carried out at 15 v. per cm. for 16 hr. at 4°C. The gel was sliced horizontally and the fresh surface was stained with Amido Black 10B in methanol-water-acetic

acid (45:45:10 v./v./v.). In the runs for glutelin subfractions, 0.5% beta-mercaptoethanol was added to the gel.

Prolamin preparations were subjected to disc electrophoresis according to the procedure of Davis (17) with a Canalco Model 6 apparatus. Protein bands were stained with Amido Black 10B. Carbohydrate bands were detected with periodic acid and Schiff's reagent.

Chemical Analyses

The carbohydrate contents of the preparations were determined by the phenol-sulfuric acid method (16). Protein concentration in solution was estimated by the Lowry method (15) with rice glutelin as standard. Protein content of lyophilized proteins was determined from micro-Kjeldahl nitrogen (10) multiplied by the factor 5.95. Double evacuation of the hydrolysis tube, according to the procedure of Kohler and Palter (18), was employed to improve the tyrosine values. Purified nitrogen was introduced into the evacuated tube before rethawing and before sealing the tube, however. The hydrolysate was analyzed in a Beckman Model 120C amino acid analyzer using the 2-hr. run with PA-28 and PA-35 resins (19). The correlation factors of Kohler and Palter (18) were applied for calculating cystine, isoleucine, methionine, serine, threonine, and valine values obtained by the standard procedure without performic oxidation. Houston et al. (20) found these factors applicable to rice protein.

RESULTS

Glutelin Extraction

A detailed study of the effect of NaOH concentration and the pH of the alkaline extract on glutelin efficiency showed that most glutelin was extracted above pH 11 (Table I). Below pH 10, the solubility of glutelin was drastically reduced as indicated by the low protein content of the 0.0075N NaOH extract. When 0.01N NaOH was used at a solvent:rice ratio of 2 ml.:g., the resulting extract had a pH of 9.8. Presumably, acidic substances that can partially neutralize the solvents are present in milled rice (21).

The phenol:acetic acid:water solvent of Jennings and Watt (22) was not an efficient extractant for rice glutelin. Other wheat gluten solvents, urea and aluminum lactate, were previously shown to be poor extractants of rice glutelin (9).

Dissociating agents such as beta-mercaptoethanol, sodium dodecyl sulfate (SDS), and urea have been used to dissolve cereal glutelins. Earlier results showed that urea was not an efficient solvent for rice glutelin (9). SDS was a better glutelin extractant than salt buffer (pH 10) with beta-mercaptoethanol (Table I). A combination of salt buffer (pH 10) with beta-mercaptoethanol and SDS extracted 83% of the rice glutelin. The extraction efficiency of the combination of beta-mercaptoethanol and SDS is much higher than that of the two solvents used in succession. In contrast, the addition of salt buffer (pH 10) to SDS improved the extraction efficiency of SDS by 7 percentage points. This improvement is similar to the extraction efficiency of the salt buffer (pH 10) solvent alone of 6%. Presumably, beta-mercaptoethanol complements SDS in dissolving rice glutelin. Cagampang et al. (1) reported 67% extraction of rice protein with 0.5% SDS. Sodium dodecyl benzene sulfonate is a good rice protein extractant only above pH

TABLE I. EXTRACTION EFFICIENCIES OF SELECTED SOLVENTS ON MILLED-RICE PROTEIN AND GLUTELIN

Solvent	pH of Extract	Extracted Protein mg.	Extraction Efficiency %
Protein[a]			
0.100N NaOH	11.8	13.1	98
0.078N NaOH	11.8	12.7	95
0.050N NaOH	11.7	12.2	91
0.025N NaOH	11.3	11.7	87
0.015N NaOH	11.1	10.7	80
0.0075N NaOH	9.8	5.3	39
0.2M Sodium carbonate-bicarbonate buffer, pH 10	10.0	2.2	17
Phenol:acetic acid:water (1:1:1 w./v./v.)	2.7	10.2	76
Glutelin[b]			
70% Ethanol with 0.6% beta-mercaptoethanol		0.49	6
0.5M NaCl with 0.1M sodium borate buffer, pH 10		0.54	6
Salt buffer (pH 10) with 0.6% beta-mercaptoethanol		0.74	9
0.5% Sodium dodecyl sulfate		3.47	42
Salt buffer (pH 10) with 0.5% sodium dodecyl sulfate		4.08	49
Salt buffer (pH 10) with 0.6% beta-mercaptoethanol and 0.5% sodium dodecyl sulfate		6.95	83

[a]Two milliliters solvent and 100 mg. BPI-76 100-mesh milled rice (13.4 mg. protein [Kjeldahl N X 5.95]) shaken for 6 hr. LSD (5%) = 1.25 mg.

[b]Two milliliters solvent and 100 mg. IR8 milled rice residue (8.33 mg. glutelin) shaken for 6 hr. LSD (5%) = 0.18 mg.

10. A 1.2% solution of the detergent alone extracted only 42% of milled-rice protein (1,23).

Glutelin Composition

A comparison of the various methods for preparing glutelin showed that most preparations had aminograms similar to those of milled-rice protein (Table II) as we previously observed (9). The only preparation which was high in lysine (6%) was the alkali extract of the method of Lózsa and Koller (12). Takeda et al. (24) also reported that one of three fractions of milled-rice glutelin is high in lysine. The high lysine content of this glutelin extract may also be due in part to its subunit interchange in the alkaline medium with the high-lysine protein, albumin, which has not been removed from the rice flour. The other glutelins with 3.5 to 4.0% lysine were extracted with alkali from milled rice which had been previously extracted with a salt solution to remove albumin-globulin and alcohol solution to remove prolamin. Our amino acid values for milled-rice protein are similar to those of Houston et al. (20) using the same analytical procedure on different rice varieties.

All the preparations contained some polysaccharides. The methods used for preparing the glutelins should have removed the free sugars. The Osborne glutelin showed the highest nitrogen content: 16.9%.

Further purification of the Osborne glutelin by the method of Sawai and Morita (13) reduced its carbohydrate content from 5.96 to 2.82% and improved its nitrogen content from 16.9 to 17.6%. No change in its aminogram was observed. Starch-gel electrophoresis in the presence of urea showed that purification removed the two faster migrating bands of the Osborne glutelin (Fig. 1). Sawai and Morita also reported a high nitrogen content (17.9%) for repurified rice glutelin.

Starch-gel electrophoresis of the rice protein fractions in the presence of urea showed that globulin has the fastest mobility followed by albumin and prolamin. Most of the glutelin remained in the sample well, but some migrating bands were also observed. Fewer albumin and globulin bands were obtained with our procedure using 12% starch gel than when a higher concentration (16 to 18%) of starch gel was used (25,26). Glutelin was only partially soluble in aluminum lactate buffer. Starch-gel electrophoresis at an alkaline pH did not improve the resolution of the proteins. Similar relative mobilities are reported for the protein fractions of wheat (14). More albumin bands were reported by Silaev et al. (27) with polyacrylamide gel at pH 8.65.

TABLE II. AMINO ACID CONTENT OF IR8 MILLED-RICE GLUTELIN PREPARATIONS

Amino Acid	IR8 Milled Rice	Glutelin Preparations			
		0.015N NaOH		0.1N NaOH	
		Lozsa	Maes	Osborne	Maes
		g./16.8 g. N		g./16.8 g. N	
Alanine	6.48	6.31	5.73	5.01	5.69
Arginine	8.73	8.72	10.7	10.6	10.2
Aspartic acid	10.8	9.87	11.2	10.8	9.81
Cystine	1.60	1.19	1.84	1.28	2.06
Glutamic acid	19.7	15.8	18.0	20.2	16.3
Glycine	4.92	5.30	5.20	4.49	4.99
Histidine	2.41	2.64	2.45	2.47	2.27
Isoleucine	4.13	5.24	4.52	4.68	4.86
Leucine	8.24	7.29	7.27	7.89	7.21
Lysine	3.80	6.21	4.02	3.50	3.48
Methionine	3.37	2.75	2.10	1.42	2.61
Phenylalanine	6.02	5.27	5.74	6.15	4.74
Proline	4.87	4.41	4.23	4.16	4.24
Serine	6.03	6.61	5.77	6.86	5.40
Threonine	4.34	4.30	4.03	3.74	3.73
Tyrosine	4.94	4.96	5.47	5.07	5.15
Valine	7.21	7.21	6.91	6.52	6.64
Ammonia	2.73	3.44	2.26	2.36	2.03
TOTAL	110.32	107.52	107.44	107.20	101.41
N recovery, %	100.5	103.9	99.0	98.4	93.3
Carbohydrate, % glucose		10.7	1.90	5.96	9.30
N content, % dry basis	1.51	14.3	13.9	16.9	10.4
Recovery, % of milled-rice protein		4.4	5.8	16.3	2.8

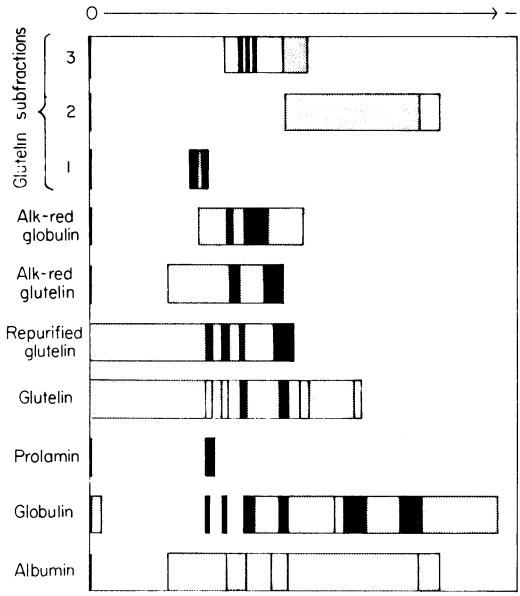

Fig. 1. Starch-gel electrophoresis in the presence of urea (aluminum lactate buffer pH 3.1) of rice protein fractions and glutelin preparations and subfractions. Stain: Amido Black 10B.

Alkylated, reduced glutelin had only two major bands (Fig. 1) of similar electrophoretic mobility to those of repurified glutelin. Most of the glutelin migrated into the starch gel after reduction in contrast with the unmodified glutelin. The reduction and subsequent alkylation of globulin, however, resulted in a preparation with a slower mobility than globulin when subjected to starch-gel electrophoresis in the presence of urea.

Gel filtration on Sephadex G-200 columns showed a second fraction with a MW equivalent to 6.5×10^4 upon reduction and alkylation of glutelin (Fig. 2). The glutelin preparations had a peak equivalent in MW to 6×10^5 as shown by gel filtration on Sepharose 4B. Hence, the reduction and alkylation resulted in a partial dissociation of glutelin into its subunits. Takeda et al. (24) reported the MW of rice glutelin fractions ranging from less than 2×10^6 to more than 20 to 30×10^6.

Subfractionation experiments were performed on the residual glutelin of IR8 milled rice after serial extraction of albumin, globulin, and prolamin. Direct extraction with tris-maleate buffer with 3M urea and beta-mercaptoethanol gave a minor fraction (1% of glutelin) with a very high lysine content of 6.4% (Table III).

Serial subfractionation of the IR8 glutelin gave three subfractions differing widely in amino acid composition (Table III). The residual protein had an aminogram similar to that of glutelin. A comparison of the aminograms of albumin, globulin, and prolamin of IR8 indicated that subfraction 1 (extracted with 70% ethanol with 0.6% beta-mercaptoethanol) was similar to prolamin in lysine content and solubility. Subfraction 2 (solvent: NaCl with beta-mercaptoethanol) had a

lysine content closest to that of globulin. Subfraction 3 (obtained with SDS) had the highest lysine content of the three subfractions. Its lysine content was closest to that of albumin. However, the contents of the other amino acids in the subfractions were not similar to those of albumin, globulin, and prolamin. Moureaux and Landry (6) also reported three similar subfractions for corn glutelin. Wheat glutelin subfractions similar to albumin in properties have also been reported (7,8).

Starch-gel electrophoresis in the presence of urea showed that glutelin subfraction 1 had two bands with similar mobilities to the single prolamin band (Fig. 1). Subfractions 2 and 3 had distinct patterns different from albumin, globulin, prolamin, glutelin, and reduced glutelin.

Prolamin

Preliminary experiments showed that 70% ethanol was a more effective solvent for prolamin than 60% ethanol, although the preparations have very similar aminograms (Table IV). Maes percolation, however, gave a prolamin with a high lysine content of 2.44%. Most preparations had low nitrogen contents. Polysaccharide analysis indicated low contents of glucan in these preparations. The preparations showed high UV absorption at 280 nm. Their Lowry protein values were much higher than their Kjeldahl protein values. Padmoyo and Högl (28) also noted that 70% ethanol was optimum for rice prolamin extraction.

Fractionation of prolamin in Sephadex G-100 columns with 0.01N NaOH as eluant gave two UV-absorbing peaks, but only the peak at the void volume (v_o) was positive for Lowry protein. Gel filtration on a Sephadex LH-20 column with 70% ethanol as eluant resulted in a partial separation of these two UV peaks. Presumably

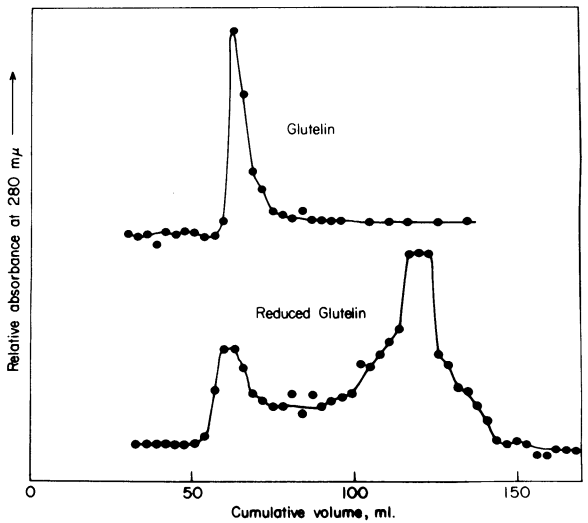

Fig. 2. Gel filtration chromatography of glutelin and alkylated reduced glutelin on a column (35 × 2.5 cm.) of Sephadex G-200.

TABLE III. AMINO ACID CONTENT OF MILLED-RICE GLUTELIN SUBFRACTIONS

		Glutelin Subfractions						
		1	2	3				
Amino Acid	IR8 Glutelin	beta-ME[a] urea pH 8	70% EtOH beta-ME[a]	Salt pH 10 Beta-ME[a]	SDS salt pH 10	Residue	Albumin	Globulin	Prolamin[b]
		g./16.8 g. N							
Alanine	5.80	5.78	5.49	6.05	6.10	6.52	8.74	9.07	6.58
Arginine	7.47	9.28	4.27	12.6	9.28	6.60	8.42	11.0	5.92
Aspartic acid	9.89	9.83	5.08	8.96	11.9	9.92	10.8	7.76	7.88
Cystine	1.48	1.12	2.78	2.66	...	1.66	2.86	...	0.30
Glutamic acid	19.8	15.8	25.5	13.1	18.7	18.8	12.5	11.8	21.4
Glycine	4.24	5.02	2.60	7.06	4.74	4.60	6.87	5.88	3.22
Histidine	2.35	2.20	1.36	2.80	2.60	2.27	2.57	1.62	0.91
Isoleucine	5.27	4.32	4.60	3.00	5.41	6.02	4.05	3.03	4.68
Leucine	8.19	6.92	8.96	5.99	8.36	9.60	7.89	6.56	11.3
Lysine	3.47	6.40	0.23	3.19	4.14	3.46	4.92	2.54	0.51
Methionine	2.61	2.45	4.34	2.17	...	3.46	2.54	2.27	0.50
Phenylalanine	5.42	4.47	5.80	3.00	6.10	6.01	2.97	3.32	6.26
Proline	4.55	4.69	4.88	5.22	4.96	6.41	6.60	5.47	4.08
Serine	6.08	7.21	6.42	4.68	6.38	6.45	5.15	5.53	5.10
Threonine	3.92	3.96	4.13	2.80	4.24	4.65	4.55	2.86	2.38
Tyrosine	4.87	4.61	6.40	3.03	3.72	3.82	3.92	4.98	8.70
Valine	7.31	6.80	6.79	6.17	7.59	8.64	8.72	6.18	6.97
Ammonia	3.47	1.76	3.18	2.90	2.75	2.72	2.05	1.55	2.98
TOTAL	106.19	102.62	102.81	95.38	107.1	111.61	106.12	91.42	99.67
N recovery, %	98.9	91.0	87.0	99.0	100.9	99	98.1	92.3	86.3
N content, % dry basis	1.59	1.38	5.70	8.74	11.8	0.38	2.11	16.8	3.57

[a]Beta-mercaptoethanol.

[b]Table IV, Osborne method, 70% ethanol solvent.

0.01N NaOH is a better solvent for dissociating protein from its UV-absorbing nonprotein contaminant than 70% ethanol. Prolamin prepared from ethanolic extracts by evaporation of the ethanol under reduced pressure, however, was not redissolved readily in 70% ethanol. The nonprotein contaminant was more soluble in ethanol than the prolamin preparation. It also was not readily dialyzable. By contrast, Landry et al. (29) reported the complete separation of the UV-absorbing nonprotein contaminant of corn prolamin by gel filtration on Sephadex LH-20.

Prolamin has a MW of 3×10^5 or approximately half the MW of glutelin from gel-filtration experiments with Bio-Gel P-300. In contrast with glutelin which was eluted at v/v_0 of 1.00, prolamin was eluted at v/v_0 of 1.45. Prolamin also migrated readily in the 12% starch gel during electrophoresis in contrast with glutelin which remained mainly in the sample well (Fig. 1). It was a slower migrating band than albumin and globulin.

A more efficient method of direct ethanol extraction was tried with IR8 and BPI-76 milled rices. The 70% ethanol solvent extracted only 2.2% (Lowry method) of IR8 protein. Water extraction prior to prolamin extraction reduced the prolamin yield to 0.74%. Extraction with 0.5M NaCl prior to prolamin extraction gave a prolamin yield of 1.0% of IR8 protein.

TABLE IV. AMINO ACID COMPOSITION OF MILLED-RICE PROTEIN AND PROLAMIN PREPARATIONS

Amino Acid	IR8 Milled Rice	IR8 Prolamin				BPI-76 Prolamin	
		Osborne		Maes	Direct extraction method	Osborne	Direct extraction method
		60% EtOH	70% EtOH	70% EtOH		70% EtOH	
		g./16.8 g. N					
Alanine	6.32	6.56	6.58	5.74	6.57	6.78	6.85
Arginine	8.78	6.19	5.92	8.38	5.29	5.94	6.64
Aspartic acid	10.8	8.02	7.88	10.3	8.33	8.16	7.90
Cystine	2.30	0.13	0.30	1.62	0.97	0.30	trace
Glutamic acid	20.1	22.2	21.4	21.0	23.3	20.8	22.5
Glycine	5.22	3.16	3.22	4.06	3.76	3.15	2.57
Histidine	2.32	0.94	0.91	4.06	1.29	1.16	1.46
Isoleucine	4.58	4.72	4.68	5.07	4.07	4.96	6.54
Leucine	8.14	12.0	11.3	8.38	10.6	10.7	13.3
Lysine	4.09	0.36	0.51	2.44	0.46	1.05	trace
Methionine	1.84	0.60	0.50	1.69	0.06	0.38	trace
Phenylalanine	5.20	6.50	6.26	6.10	5.78	5.70	6.60
Proline	3.52	3.98	4.08	4.53	4.38	4.08	4.62
Serine	4.98	4.73	5.10	5.85	6.91	5.57	6.33
Threonine	4.36	2.24	2.38	2.54	3.13	2.52	2.51
Tyrosine	4.74	9.32	8.70	5.99	7.78	7.68	9.83
Valine	7.87	6.78	6.97	7.50	5.64	6.07	6.01
Ammonia	3.40	3.00	2.98	2.32	4.52	3.20	3.65
TOTAL	108.6	101.5	99.67	108.6	102.8	98.20	107.3
N recovery	101.7	86.9	86.3	94.2	89.1	87.0	104.0
N content, % dry basis	1.81	8.00	3.57	12.4	1.85	3.88	16.7
Polysaccharide, % dry basis		4.4	5.7	2.3	2.8	7.2	0.31

Acetone precipitation of prolamin was readily achieved with BPI-76 (0.66 mg. protein per ml.) but not with IR8 (0.33 mg. per ml.). After dilution of the BPI-76 extract to the same protein concentration as IR8 with 70% ethanol, the BPI-76 prolamin was no longer precipitated by acetone addition. Salt addition did not enhance prolamin precipitation. The precipitated BPI-76 prolamin had a high nitrogen content of 16.7%. The prolamin from IR8 was also precipitated by ethanol evaporation, redissolved in 0.1N NaOH, and subjected to ultrafiltration. Gel filtration of IR8 ultrafiltrate and protein concentrate on Sephadex G-100 column revealed that the nonprotein contaminant was only incompletely separated from the prolamin.

Acetone precipitation was the easiest way to get pure prolamin from the direct ethanol extraction of milled rice. Disc electrophoresis, gel filtration, and amino acid analysis (Table IV) showed that the direct ethanol extract was the same as the prolamin extracted after removal of albumin and globulin.

Disc electrophoresis indicated the presence of two prolamin protein bands which also stained for carbohydrates, Presumably some glycoproteins may be present in rice prolamin as a minor fraction.

Colorimetric tests on the ultrafiltrate indicated that the contaminant was polyphenolic in nature. Phenols absorb light in the UV region, interfere with the Lowry protein test, and form a complex with protein which may be dissociated at pH >10 (30). The contaminant gave a yellow color with ethanolic NaOH and a yellow-orange to wine-red color with concentrated sulfuric acid. These are general tests for flavonoid compounds and quinones (31). The UV spectrum of the contaminant showed a sharp maximum at 250 nm. in 0.01N NaOH and another minor peak was observed at 340 to 380 nm. in 0.1N NaOH. Paper chromatography with 2-propanol-ammonia-water (8:1:1 v./v./v.) as developer (32) showed a yellow fluorescing spot with an R_f value of 0.05 to 0.06 with tailing up to the sample origin.

DISCUSSION

Glutelin

Comparison of various extraction procedures showed that the solution of SDS with beta-mercaptoethanol and 0.5M NaCl in sodium borate buffer (pH 10) was an effective extractant of rice glutelin (Table I). Although the glutelin is broken down into subunit proteins by the solvent, and the detergent is difficult to separate from its complex with protein, such a preparation could be used for amino acid analysis and electrophoretic studies.

Sodium hydroxide at pH 10 or lower is a poor solvent of rice glutelin. A study in which NaOH extracts were ultracentrifuged at 59,780 r.p.m. showed two or three fractions depending on protein and alkali concentration (21). The third, higher MW fraction was observed only at lower NaOH concentrations and at higher protein concentrations of the extracts studied. Dilution of the protein extracts with solvent also made the third peak disappear. Hence, dissociation of glutelin into smaller subunits due to charge repulsion occurs at high alkali concentrations or at low protein concentrations.

Repurification of glutelin did not completely eliminate its few migrating bands in the starch-gel electrophoregram. These probably are subfractions of glutelin that are soluble in aluminum lactate buffer rather than contaminant proteins. The contaminant proteins should have been removed by the purification method of precipitation from NaCl solutions and repeated washing with this solvent. Takeda et al. (24) also reported the heterogeneity of rice glutelin since they obtained three fractions by gel filtration on Sepharose 2B.

Reduction and alkylation of glutelin were also incomplete, since the reaction was essentially heterogeneous. A fraction with a MW one-tenth that of the native glutelin was obtained. Sawai and Morita (13) also found one major fraction of alkylated, reduced rice glutelin. The two minor fractions of their glutelin derivative may be contaminant proteins.

Serial extraction with solvents containing beta-mercaptoethanol or SDS showed that rice glutelin is made up of protein subunits differing widely in properties. The high MW of glutelin thus is due to disulfide bond cross-linkages between its protein subunits and to hydrophobic bonding. The former are broken by beta-mercaptoethanol and the latter by SDS. These results are similar to those obtained for corn glutelin (6) and for alkali-soluble wheat proteins (7,8). The

decrease in albumin-globulin level in the grain during maturation that we observed previously (9) must have been due to disulfide and hydrophobic bonding which reduced the solubility of the resulting protein.

Prolamin

One reason little work has been done on rice prolamin is its low content in the grain. In addition, the procedure requires the removal of albumin-globulin before prolamin is extracted with aqueous ethanol. Previous results of paper chromatography of aqueous ethanol extracts of milled rice by Padmoyo and Högl (28) indicated that albumin is absent in the 70% ethanol extract. Our results showed that prolamin can be extracted directly from milled rice. Recently kaffirin from sorghum grains was prepared similarly (33).

The low nitrogen content of prolamin indicates nonprotein contamination. Since the contaminant could not be dialyzed, it must form a complex with the prolamin or it may have a similar MW as prolamin. Since the contaminant had a high UV absorption and its Lowry protein was higher than its micro-Kjeldahl protein level, it must be polyphenolic. Concentrating the ethanolic extract of prolamin by ultrafiltration or by removal of the ethanol fraction of the solvent promoted either the complexing of prolamin with polyphenols and their oxidized forms or a change in conformation of the prolamin. The resulting prolamin-polyphenol complex or denatured prolamin was less soluble in 70% ethanol and was solubilized only by dilute alkali.

The simplest method of separating prolamin from polyphenolic compounds was precipitation of prolamin from the alcoholic extract with acetone. However, the concentration of prolamin in the 70% ethanol extract was critical for effective precipitation with acetone. The immediate separation of prolamin from the polyphenols prevents the formation of stable complexes with oxidized polyphenols (30). Acetone precipitation was also employed by Meredith (34) to isolate cereal prolamins from methanol-chloroform (1:2 v./v.). We previously found, however, that the methanol-chloroform extract has a lower MW ($<10^4$) than the 70% ethanol extract of milled rice (26).

Our studies demonstrated the heterogeneity of preparations of rice glutelin and prolamin and the artificiality of grouping these proteins in distinct solubility classes.

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